Synthesis of potentially-bioactive lactosyl-oligofructosides by a novel bi-enzymatic system using bacterial fructansucrases

Marina Díez-Municio[a], Clara González-Santana[a], Blanca de las Rivas[b], M. Luisa Jimeno[c], Rosario Muñoz[b], F. Javier Moreno[a*] and Miguel Herrero[a]

(a) Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), CEI (UAM+CSIC), C/ Nicolás Cabrera 9, 28049 Madrid, Spain.

(b) Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN (CSIC), C/ Juan de la Cierva 3, 28006 Madrid, Spain.

(c) Centro Química Orgánica "Lora-Tamayo" (CSIC), C/ Juan de la Cierva 3, 28006 Madrid, Spain.

* Corresponding author: Tel.: +34 91 0017948; E-mail address: javier.moreno@csic.es

Abstract

Efficient enzymatic synthesis of lactosyl-oligofructosides (LFOS) with degree of polymerization from 4 to 8 was achieved in the presence of sucrose:lactosucrose and sucrose:lactose mixtures by transfructosylation reaction. The main synthesized LFOS which consist of β-2,1-linked fructose to lactosucrose: β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-[(1→2)-β-D-fructofuranosyl]n-(1→2)-β-D-fructofuranoside (where n refers to the number of transferred fructose moieties) were structurally characterized by nuclear magnetic resonance (NMR). The maximum formation of LFOS was 81% (in weight respect to the initial amount of lactosucrose) and was obtained after 24 h of transfructosylation reaction based on sucrose:lactosucrose (250 g L^{-1} each) catalyzed by an inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS). The production of LFOS in the presence of sucrose:lactose mixtures required a previous high-yield lactosucrose synthesis step catalyzed by using a levansucrase from *Bacillus subtilis* CECT 39 (LS) before the inulosucrase-catalyzed reaction. This novel one-pot bi-enzymatic system led to the synthesis of about 22% LFOS in weight, with respect to the initial amount of lactose (250 g L^{-1}). The results revealed a high specificity for the substrate involved in the inulosucrase-catalyzed reaction given that, although lactosucrose (*O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) acted as a strong acceptor of β-2,1-linked fructose, lactose (β-D-galactopyranosyl-(1→4)-α-D-glucose) was found to be an extremely weak acceptor.

Keywords: lactosyl-oligofructosides; lactosucrose; inulosucrase; levansucrase; bi-enzymatic system; prebiotic; transfructosylation.

Chemical compounds studied in this article

Lactosylfructoside (PubChem CID: 174626).

1. Introduction

Lactose (*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose) and sucrose (*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) are widely recognized as suitable cheap sources for the production of bioactive carbohydrates and derivatives through enzymatic processes (Díez-Municio, Herrero, Olano, & Moreno, 2014). The most important product obtained as a result of an enzymatic transfer reaction between both disaccharides is the trisaccharide lactosucrose (*O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), also named lactosylfructoside suggesting that lactose acts as acceptor of the fructosyl moiety of sucrose (transfructosylation) or galactosylsucrose remarking that galactose moiety of lactose (donor) is transferred to sucrose through transgalactosylation by β-galactosidase (EC 3.2.1.23) from *Bacillus circulans* (Li et al., 2009). Transfructosylation reaction may occur either by β-fructofuranosidase (EC 3.2.1.26) from *Arthrobacter sp.* K-1 (Fujita, Hara, Hashimoto, & Kitahata, 1990; Pilgrim et al., 2001) or levansucrases (EC 2.4.1.10) from various bacteria strains, among which *Bacillus subtilis* enzyme exhibits an effective lactosucrose-producing ability (Park, Choi, & Oh, 2005). In the way of finding the most ideal enzyme for industrial lactosucrose production, the use of recombinant levansucrases has also been explored (Han et al., 2009; Seibel et al., 2006) which ensures an adequate enzymatic supply and contributes to improved product quality.

Since the advent of lactosucrose industrial production in the 1990's (Fujita, Hara, Hashimoto, & Kitahata, 1992), different potential beneficial effects have been associated to this carbohydrate, reported mainly by Japanese researchers (Mu, Chen, Wang, Zhang, & Jiang, 2013). The most-notable potential effects of lactosucrose include: prebiotic properties to modulate the intestinal microbiota due to its ability to enhance the growth of beneficial bifidobacteria (Fujita, Ito, & Kishino, 2009; Ohkusa, Ozaki, Sato, Mikuni, & Ikeda, 1995); calcium absorption-promoting activity (Fujita et

al., 2006; Teramoto et al., 2006); and, potential use as a low-calorie sweetener because of being poorly digested in the human small intestine (Côté, 2007). This non-digestible trisaccharide might gain further worldwide recognition as a prebiotic ingredient if its ingestion is linked to lactosucrose-derived oligosaccharides with higher degree of polymerization, such as the well-known prebiotic fructo-oligosaccharides (FOS), since longer carbohydrate chains normally possess slower fermentation rates (Perrin, Fougnies, Grill, Jacobs, & Schneider, 2002).

However, to the best of our knowledge, there is only one method described in the literature for the preparation of fructosyl oligosaccharides derived from lactosucrose; Yamamori, Onodera, Kikuchi, & Shiomi (2002) reported the enzymatic production of mono and difructosyl-lactosucrose by a purified enzyme obtained from asparagus roots (*Asparagus officinalis* L.). In that approach, trisaccharides kestose and lactosucrose were employed as substrates. This method has the main disadvantage of a low production efficiency (11%, in weight respect to the initial amount of the donor saccharide, after 48 h of process reaction). In addition, the obtained oligosaccharides are not synthesized with high purity due to the large amount of substrates that remain unhydrolyzed (kestose and lactosucrose), as well as due to the formation of other derived products such as sucrose and nystose. Moreover, the use of expensive and not readily available raw materials makes its industrial production hardly feasible. Finally, a small amount of toluene, a highly toxic reactive incompatible with human consumption, is required. In this context, the present work addresses the high-yield production and subsequent nuclear magnetic resonance (NMR)-based structural characterization of potentially bioactive fructosyl oligosaccharides derived from lactosucrose (lactosyl-oligofructosides, LFOS) by the straightforward transfructosylation of lactosucrose catalyzed by an inulosucrase (IS) from *Lactobacillus gasseri* DSM 20604. Furthermore,

the production of LFOS from readily available and inexpensive sources such as sucrose and lactose through a bi-enzymatic system requiring a previous high-yield lactosucrose synthesis step catalyzed by a levansucrase from *Bacillus subtilis* CECT 39 is also reported.

2. Materials and methods

2.1. Chemicals, reagents and carbohydrates.

All chemicals and reagents used were of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany), VWR (Barcelona, Spain) and Merck (Darmstadt, Germany). Fructose, glucose, sucrose and lactose were purchased from Sigma–Aldrich (Steinheim, Germany) and lactosylfructoside, nystose and 1^F-fructofuranosylnystose from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Production and purification of recombinant inulosucrase and levansucrase enzymes.

A fragment of the recombinant inulosucrase (IS) protein lacking the cell-anchoring-motif from *Lactobacillus gasseri* DSM 20604 (Anwar et al., 2010) was overproduced in *Escherichia coli* and purified as described by Díez-Municio et al. (2013). Levansucrase (LS) (accession YP_003867730) from *Bacillus subtilis* CECT 39 (ATCC 6051) was overproduced in *Escherichia coli* and purified. Briefly, a 1.3 kb *sacB* DNA fragment, lacking the 29 N-terminal amino acids corresponding to the signal peptide, was PCR-amplified by HS Primer Start DNA polymerase (Takara) using the primers 1088 (5′-TAACTTTAAGAAGGAGATATACAT**atg**gaaacgaaccaaaagccgtata) and 1089 (5′-GCTA**TTA***ATGATGATGATGATGATG*tttgttaattgttaattgtccttg) (the nucleotides pairing the *sacB* gene sequence are written in lowercase letters; the start and

117 stop codons are indicated in bold; the nucleotides coding the His_6 tag are written in
118 italics). The 1.3 kb purified PCR product was inserted into the pURI3-Cter vector using
119 a restriction enzyme-free and ligation-free cloning strategy (Curiel, de las Rivas,
120 Mancheño, & Muñoz, 2011). The cloning of a protein into the pURI3-Cter expression
121 vector introduced a His_6-tag on the C-terminus of the recombinant protein produced.
122 Thus, the final recombinant His_6-tagged LS protein consisted of 450 amino acid
123 residues with a molecular weight of 50 kDa. *E. coli* DH10B cells were transformed and
124 recombinant plasmids containing the correct insert (pURI3-Cter-LS) were isolated. For
125 protein production, cells carrying pURI3-Cter-LS plasmid were grown at 37 ºC until
126 they reached an optical density of 0.4 at 600 nm, and induced by adding IPTG (0.4 mM
127 final concentration). After induction, the cells were grown at 22 ºC during 20 h and
128 collected by centrifugation. Cells were resuspended in phosphate buffer (50 mM, pH 7.0
129 containing 300 mM NaCl). Crude extracts were prepared by French press lysis of the
130 cell suspension (three times at 1100 psi). The insoluble fraction of the lysate was
131 removed by centrifugation at 47,000 g for 30 min at 4 ºC. The supernatant was filtered
132 through a 0.45 μm filter and applied to a TALON®Superflow™ histidine affinity resin
133 equilibrated with 50 mM sodium phosphate pH 7.0, 300 mM NaCl and 10 mM
134 imidazole to improve the interaction specificity in the affinity chromatography step. The
135 bound LS enzyme was eluted using 150 mM imidazole in the same buffer. The eluted
136 protein was dialyzed against 50 mM sodium phosphate, pH 7.0 containing 300 mM
137 NaCl for 20 h at 4ºC. The purity of the LS protein was determined by 12.5% (w/v)
138 SDS-PAGE at the various stages of the purification process and is shown in **Fig. S1**
139 (supplemental material).
140

141 ***2.3. Enzyme characterization: inulosucrase (IS) and levansucrase (LS) activity***
142 ***assay.***

143 The protein concentration of the purified IS and LS was 16.2 and 8.7 mg mL^{-1},
144 respectively, according to the bicinchoninic acid (BCA) assay using as standard a
145 dextransucrase of *Leuconostoc mesenteroides* B-512F purchased from CRITT Bio-
146 Industries (Toulouse, France). Fructosidase and fructosyltransferase activities of IS and
147 LS were measured as a function of the amounts of glucose and fructose released from a
148 solution of sucrose (100 g L^{-1}) according to Díez-Municio et al. (2013). IS expressed a
149 specific fructosidase activity of 17.4 units per milligram (U mg^{-1}), where 1 unit is
150 defined as the amount of enzyme releasing 1 μmol of glucose per minute per milliliter
151 under the assayed conditions. The fructosyltransferase activity was 10.5 U mg^{-1}, where
152 1 unit is defined as the amount of enzyme required to transfer 1 μmol of fructose per
153 minute at other molecules under the assayed conditions. Meanwhile, LS expressed a
154 specific fructosidase activity of 2.9 U mg^{-1} and the fructosyltransferase activity was 1.9
155 U mg^{-1}.
156
157 ***2.4. Enzymatic synthesis of lactosyl-oligofructosides (LFOS).*** The production
158 of LFOS in the presence of sucrose (fructosyl donor) and lactosucrose (acceptor) (250 g
159 L^{-1} each) through transfructosylation reaction catalyzed by the *L. gasseri* IS was carried
160 out under the reaction conditions (IS concentration, pH, temperature) previously
161 established for the synthesis of fructo-oligosaccharides (FOS) and maltosyl-fructosides
162 (MFOS) (Díez-Municio et al., 2013): 1.6 U of IS per mL, pH 5.2 (25 mM sodium
163 acetate buffer, supplemented with 1 mM $CaCl_2$) and 55ºC. Samples were incubated in
164 individual tubes of 1.5 mL in an orbital shaker at 1000 rpm. The reactions were allowed
165 to proceed up to 48 h. Aliquots were taken from the reaction mixture at suitable time

intervals (1, 3, 8, 24, 32 and 48 h). The enzyme was inactivated by heating at 100 ºC for 5 min and inactivated samples were then diluted with acetonitrile:water (40:60, *v:v*), filtered using a 0.45 µm syringe filter (Symta, Madrid, Spain), and analyzed by LC-RID. Results are shown as mean ± sd of triplicate assays.

Furthermore, the production of LFOS was studied using sucrose and lactose as acceptor (250 g L^{-1} each) by a patented bi-enzymatic system (Moreno et al., 2014) requiring a lactosucrose synthesis step catalyzed by a *B. subtilis* LS prior to the inulosucrase-catalyzed reaction. Lactosucrose production was optimized in 50 mM potassium phosphate buffer (pH 5.0, 6.0 and 7.0) at four assayed working temperatures (30, 37, 45 and 55°C) and two enzyme concentrations (0.25 and 0.5 U mL^{-1}). Aliquots were taken at suitable time intervals (0, 1, 2, 3, 8 and 24 h). The reaction was stopped by heating at 100 ºC for 5 min and inactivated samples were then prepared to LC-RID analysis. Results are shown as mean ± sd of triplicate assays. Once reaction conditions for lactosucrose synthesis were optimized, the subsequent transfructosylation reaction catalyzed by the *L. gasseri* IS was allowed to proceed up to 24 h, taking also aliquots at 3 and 18 h.

2.5. Chromatographic determination of carbohydrates by liquid chromatography with refractive index detector (LC-RID).

Enzymatic reactions were monitorized by LC-RID on an Agilent Technologies 1220 Infinity LC System – 1260 RID (Boeblingen, Germany) as indicated by Díez-Municio et al. (2013). Quantitative analysis was performed by the external standard method, using calibration curves in the range 0.01 - 10 mg for glucose (quantification of monosaccharides), sucrose and lactose (quantification of disaccharides), lactosucrose (quantification of trisaccharides), nystose (quantification of tetrasaccharides) and 1^F-

fructofuranosylnystose (quantification of pentasaccharides and acceptor products of polymerization degree above 5). All analyses were carried out in triplicate.

2.6. Purification and structural characterization of lactosyl-oligofructosides (LFOS) by nuclear magnetic resonance (NMR).

Considering the lack of commercially available standard for LFOS, the main oligosaccharides (DP 4-6) synthesized after 24 h of transfructosylation reaction based on sucrose:lactosucrose mixture catalyzed by IS under the optimized conditions described above were isolated and purified by preparative LC-RID on an Agilent Technologies 1260 Infinity LC System (Boeblingen, Germany) using a Zorbax NH_2 PrepHT preparative column (250 × 21.2 mm, 7 μm particle size) (Agilent Technologies, Madrid, Spain). Two mL of reaction mixtures (200 mg of total carbohydrates) were eluted with acetonitrile:water (65:35, *v:v*) as the mobile phase at a flow rate of 21.0 mL min^{-1} for 30 min. The separated LFOS were collected using an Agilent Technologies 1260 Infinity preparative-scale fraction collector (Boeblingen, Germany), and the fractions were pooled, evaporated in a rotatory evaporator R-200 (Büchi Labortechnik AG, Flawil, Switzerland) below 25 ºC and freeze-dried. Structure elucidation of the purified oligosaccharides was accomplished by NMR. NMR spectra were recorded at 298 K, using D_2O as solvent, on a Varian SYSTEM 500 NMR spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5-mm HCN cold probe. Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in parts per millon were determined relative to an internal standard of sodium [2, 2, 3, 3-$^2H_4$]-3-(trimethylsilyl)-propanoate in D_2O (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) in D_2O, respectively. One-dimensional (1D) NMR experiments (^1H, and ^{13}C) were performed using standard Varian pulse sequences. Two-dimensional (2D) [^1H, ^1H] NMR experiments [gradient correlation spectroscopy (gCOSY), total correlation

spectroscopy (TOCSY), and rotating-frame Overhauser effect spectroscopy (ROESY)] were carried out with the following parameters: delay time of 1 s, spectral width of 1179.2 Hz in both dimensions, 4096 complex points in t2, 4 transients (16 for ROESY) for each of 128 time increments, and linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. 2D [$^{1}H-^{13}C$] NMR experiments [gradient heteronuclear single-quantum coherence (gHSQC) and gradient heteronuclear multiple-bond correlation (gHMBC)] used the same ^{1}H spectral window, a ^{13}C spectral window of 30165 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively.

3. Results and discussion

3.1. Enzymatic synthesis of lactosyl-oligofructosides (LFOS) derived from sucrose:lactosucrose mixtures by L. gasseri DSM 20604 inulosucrase (IS).

Assays conducted to production of lactosyl-oligofructosides (LFOS) were initially carried out in the presence of sucrose:lactose mixtures (250 g L^{-1} each substrate) by using the purified *L. gasseri* DSM 20604 inulosucrase (IS). This was based on the results previously obtained for the enzymatic synthesis of fructooligosaccharides (FOS) and maltosyl-fructosides (MFOS) derived from sucrose and sucrose:maltose mixtures (Díez-Municio et al., 2013). In analogy to these previous experiments, the reaction was allowed to proceed up 48 h by incubating 1.6 U of IS per mL at 55 ºC in 25 mM sodium acetate buffer with 1 mM $CaCl_2$ (pH 5.2) (established as optimum reaction conditions). Nevertheless, in this case, the capacity of IS to transfer fructose units (from sucrose) to lactose (acting as acceptor) was very limited, even when a higher enzyme charge was tested (3.2 U mL^{-1}). Sucrose was gradually consumed (the

241 more enzyme charge, the faster) producing a mixture of inulin-type FOS with various
242 degrees of polymerization (DP) comprised of β-2,1-linked fructose to sucrose (thus,
243 acting both as donor and acceptor). As we previously inferred (Díez-Municio et al.,
244 2013), by comparing the retention times (t_R) with those of available commercial
245 standards, the trisaccharide 1-kestose (GF2) was the main FOS formed, followed by
246 nystose (GF3) and fructosylnystose (GF4). However, it can be observed that lactose
247 remained almost constant **(Fig. 1A)** resulting in a reaction product mixture extremely
248 poor in lactose-derived oligosaccharides.

249 Nonetheless, in an effort to obtain LFOS by IS, similar experiments were
250 performed using the trisaccharide lactosucrose (β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-
251 Fru) instead of lactose (β-D-Gal-(1→4)-D-Glc). When lactosucrose was present along
252 with sucrose in the reaction mixture, a series of new oligosaccharides suspected to be
253 composed of a lactosucrose molecule linked to fructosyl residues (termed as LFOS)
254 appeared as the reaction proceeded (**Fig. 1B**). A substantial reduction of the previously
255 identified synthesized FOS was observed. A significant decrease of lactosucrose, i.e. up
256 to a 44% after 24 h of reaction, was also indicative of the formation of these new
257 oligosaccharides based on the transfer of fructose moieties (from the donor saccharide,
258 sucrose) to lactosucrose (acceptor).

259 **Fig. 2** shows a representative LC-RID profile of the synthesized
260 oligosaccharides from a 25:25 sucrose:lactosucrose mixture after 24 h of reaction.
261 Whereas glucose (peak 2) derived from the hydrolysis of sucrose (peak 3) was released,
262 fructose (peak 1) moieties were transferred either to other sucrose molecules, producing
263 FOS of various DP (peaks 6, 8, 10, 12, 14, and 16) or, specially, to lactosucrose (peak
264 7) leading to a series of new peaks termed as LFOS (peaks 9, 11, 13, 15, and 17). As
265 can be clearly appreciated in **Fig. 2**, the efficiency of the acceptor reaction decreased as

266 the size of the LFOS chain increased. **Table 1** shows the highest content in LFOS from
267 DP4 to DP7 attained after 24 h of reaction, i.e. 202.7 mg mL^{-1} of total LFOS (81% in
268 weight respect to the initial amount of lactosucrose), clearly favored against the
269 formation of FOS (49.1 mg mL^{-1}). The productivity determined after the first hour of
270 reaction was 98 g L^{-1} h^{-1}.

271 Following the same pattern than that found for the synthesis of FOS, it could be
272 expected that LFOS were mainly comprised of β-2,1-linked fructose to lactosucrose.
273 These results indicated the capacity of IS to transfer fructosyl residues from sucrose to
274 the fructose unit of the trisaccharide lactosucrose but not to the glucose moiety of
275 lactose. Taking into account that the orientation of the incoming acceptor substrate
276 determines the type of glycosidic bond of the product (Pijning, Vujičić-Žagar, Kralj,
277 Dijkhuizen, & Dijkstra, 2012), it is suggested that lactosucrose could enter the IS
278 acceptor site by its sucrose moiety, considering that sucrose is the preferred donor and
279 acceptor of inulosucrase-type enzymes.

280 Since lactosucrose , as its own name suggest, is a derivative from lactose and
281 sucrose, the possibility that this substrate was acting both as donor and as acceptor in
282 the inulosucrase-catalyzed reaction, was also studied. However, when lactosucrose was
283 the only starting saccharide present in the reaction mixture, neither FOS nor LFOS
284 synthesis was found (data not shown). This indicates that although IS has the capacity to
285 hydrolyze sucrose, this ability is not present when the sucrose molecule is bound to a
286 galactose residue by a β-(1→4) linkage. In this sense, Ozimek, Kralj, van der Maarel,
287 & Dijkhuizen (2006) described the mode of action of fructansucrases based on the
288 double-displacement reaction mechanism. Firstly, sucrose enters the active centre of the
289 enzyme between the amino acid residues located at the subsites −1 and +1 (according to
290 the nomenclature proposed by Davies, Wilson, & Henrissat, 1997) and the glycosidic

linkage of sucrose is cleaved to give a covalent fructosyl-enzyme intermediate formed at −1 whereas glucose is released. In the second step, another acceptor substrate may enter the active site and bind to the +1 and +2 subsites and react with the fructosyl-enzyme intermediate at −1, resulting in the oligosaccharide formation. These authors explained that the fructosyltransferase −1 subsite is highly specific for accommodating fructose units, whereas the +1 subsite seems to be more flexible, exhibiting affinity for both glucose and fructose. Our results seem to confirm this finding which could explain the fact that lactosucrose acts as an effective acceptor for oligosaccharide formation in inulosucrase-catalyzed reactions but not as a donor substrate.

Finally, due to the lack of available standards of LFOS, an exhaustive NMR characterization of the most abundant LFOS (peaks 9, 11, and 13, **Fig. 2**) was further carried out to fully elucidate their structures as it will be shown below.

3.2. Enzymatic synthesis of lactosyl-oligofructosides (LFOS) derived from sucrose:lactose mixtures through a new bi-enzymatic system with B. subtilis CECT 39 levansucrase (LS) and L. gasseri DSM 20604 inulosucrase (IS).

Once known that LFOS production was feasible from sucrose:lactosucrose mixtures by using the purified IS, we studied the possibility of producing LFOS from sucrose:lactose mixtures. This new approach included a first step which involved the synthesis of lactosucrose with a levansucrase from *Bacillus subtilis* CECT 39 (LS) in order to make the synthesis of LFOS much more cost-effective. Thus, first assays for the production of LFOS using the disaccharides sucrose and lactose as substrates (250 g L^{-1} each) were aimed towards the efficient synthesis of lactosucrose. Initial conditions were based on those used by Canedo et al. (1999) (i.e., 37ºC, pH 6.0, potassium phosphate buffer 50 mM) for the production of the trisaccharide erlose also with a

316 levansucrase from *Bacillus subtilis*. Further optimization was performed following an
317 univariate method with the aim to study first the influence of different enzymes charges
318 (0.25 and 0.5 U mL^{-1}), then pH values (5.0, 6.0 and 7.0) and finally the temperature of
319 incubation (30, 37, 45 and 55°C) (**Fig. 3A**, **B**, and **C**, respectively). From all these
320 experiments, it could be concluded that the most favorable conditions included the use
321 of 0.5 U mL^{-1} of LS, pH 6.0 and 37ºC. Under these optimum reaction conditions a
322 production of 208 g L^{-1} equivalent to a yield of 82% of lactosucrose (in weight respect
323 to the initial amount of lactose) was achieved after 90 min of reaction leading to a
324 productivity value of 139 g L^{-1} h^{-1}. The yield and productivity of lactosucrose reported
325 in this work is particularly high in comparison to most of the values described in the
326 literature and which were recently reviewed by Mu, Chen, Wang, Zhang, & Jiang
327 (2013). Park, Choi, & Oh (2005), who tested various bacteria containing levansucrase
328 activity to synthetize lactosucrose, reported a similar yield by using a strain from
329 *Bacillus subtilis*, although it was necessary to keep the enzymatic reaction for 10 h
330 which remarkably impaired lactosucrose productivity as compared to the results
331 reported here.

332 Once lactosucrose production by LS was optimized, we proceeded to add the IS
333 enzyme to the reaction mixture with the objective of synthesizing LFOS through a bi-
334 enzymatic system LS-IS. The carbohydrate composition after the incubation with the
335 LS (inactivated by heating at 100ºC for 5 min) was comprised of 208 mg mL^{-1} of
336 lactosucrose, 154 mg mL^{-1} of lactose, 135 mg mL^{-1} of sucrose, 67 mg mL^{-1} of glucose
337 and 3 mg mL^{-1} of fructose. After 18 h of incubation with the IS, the synthesis of 22%
338 LFOS (in weight respect to the initial amount of lactose) was attained. Quantitative data
339 of the synthesized LFOS and FOS are summarized in **Table 1**. Since, it is well known
340 that an increase in the concentration of substrates normally leads to higher acceptor

reaction efficiency (Côté, 2007), additional synthesis studies adding extra sucrose (7 and 12% *w/v*) (keeping the lactosucrose concentration at 20% *w/v*) were performed in order to increase LFOS yield. However, a higher concentration of sucrose mainly favored the formation of FOS (data not shown) and, consequently the LFOS purity was reduced.

An interesting benefit of applying the bi-enzymatic system LS-IS (**Fig. 4A**) is the generation of potential prebiotic LFOS with relatively high yield and purity from readily available and inexpensive sources such as sucrose and lactose. Moreover, this method could allow the reuse of food-related by-products, since both raw materials are present in high amounts in important agro-industrial residues, such as cheese whey permeate and beet or cane molasses (Vandamme, 2009). Furthermore, although the efficiency of the acceptor reaction is clearly favored when lactosucrose is present as starting material, the proportion of synthesized LFOS and FOS obtained with lactose as substrate was even slightly leaned towards the formation of LFOS (**Table 1**).

Since these synthesized LFOS can be considered both lactosucrose and FOS derivatives, they might provide a good opportunity for a range of potential applications in the food industry. Focusing on bioactive properties, lactosucrose and its fructosyl derivatives could potentially be employed as prebiotic oligosaccharides that could effectively combine the healthy benefits attributed to lactosucrose and the possibility to possess lower fermentation rates as they share structural features with the β-2,1-linked FOS. Nonetheless, it would be necessary to address further *in vitro* and *in vivo* bioactivity studies, especially those related to explore if the molecular size may affect the digestion rate, as well as the fermentation speed and the metabolites produced in the gut. In this context, it has been reported that oligosaccharides of longer chains, resisting at least partially the gastrointestinal digestion, are generally fermented slowly increasing

its capacity to reach the distal regions of the colon (Harmsen, Raangs, Franks, Wildeboer-Veloo, & Welling, 2002; Tuohy, Finlay, Wynne, & Gibson, 2001) where the incidence of chronic gut disorders is higher (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004).

3.3. Structural elucidation of novel lactosyl-oligofructosides (LFOS) by nuclear magnetic resonance (NMR).

Three chromatographic peaks (9, 11 and 13, **Fig. 2**) were purified by preparative LC-RID and subsequently successfully characterized by NMR (structures **B**, **C**, **D**, respectively, **Fig. 5**). **Fig. 5B-5D** show the structures elucidated by the combined use of 1D and 2D [^{1}H, ^{1}H] and [^{1}H, ^{13}C] NMR experiments (gCOSY, TOCSY, multiplicity-edited gHSQC and gHMBC). ^{1}H and ^{13}C NMR chemical shifts observed are summarized in **Table 2**. Full set of spectra are available in the supplemental material (**Fig. S2-S16**). The 1D ^{1}H NMR spectrum of **B** showed two resonances in the anomeric region (δ5.28 and δ4.33), and besides 1D ^{13}C NMR spectrum showed signals corresponding to 24 carbons including four anomeric carbons (δ106.49, δ106.04, δ105.67 and δ94.99), indicative of the presence of a tetrasaccharide with four hexose sugars in the structure. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances. So, the anomeric carbon at δ105.67 correlated with a beta anomeric proton at δ4.33 (J(H1,H2)=7.9 Hz) and the anomeric carbon at δ94.99 correlated with an alpha anomeric proton at δ5.29 (J(H1,H2)=3.9 Hz). The anomeric carbons at δ106.49 and 106.04 were quaternary carbons. In addition, six methylene carbons at δ65.07, δ64.91, δ63.80, δ63.58, δ63.19 and δ62.26 were identified. The ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY experiments revealed the ^{1}H signals of galactopyranose, glucopyranose and fructofuranose residues (**Fig. 5**).

391 The ^{1}H-^{1}H ROESY experiment showed correlations between the H2 and H1 methylene
392 protons for the two fructose units. From these data we determined that the
393 tetrasaccharide consisted of a unit of β-galactopyranose, a unit of α-glucopyranose, and
394 two units of β-fructofuranose.

395 The position of glycosidic linkages was analyzed as follows. gHMBC showed
396 correlations between the β-Gal-C1 anomeric carbon (105.67 ppm) and α-Glu-H4 proton
397 (3.57 ppm), between the α-Glu-H1 anomeric proton (5.28 ppm) and one of the β-Fru
398 anomeric carbons (106.04 ppm), and between the β-Fru-H1 methylene protons (δ3.64,
399 and δ3.56) and the other β-Fru anomeric carbon (106.49ppm). So, the main synthesized
400 LFOS (peak 9, **Fig. 2**) was identified as the tetrasaccharide β-D-galactopyranosyl-
401 (1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside
402 which can be denominated as fructosyl-lactosucrose (**Fig. 5B**).

403 1D ^{1}H NMR spectra of **C** and **D** showed also two doublets in the anomeric
404 region. The corresponding 1D ^{13}C NMR spectra showed five (δ106.42, δ106.00,
405 δ105.80, δ105.67, and δ95.00) anomeric carbons for **C**, and six (δ106.45, δ106.00,
406 δ105.81, δ105.79, δ105.67, and δ95.00) anomeric carbons for **D**, consistent with the
407 presence of a penta- and a hexasaccharide respectively. From these data we determined
408 that the pentasaccharide consisted of a unit of β-galactopyranose, a unit of α-
409 glucopyranose, and three units of β-fructofuranose. The hexasaccharide consisted of a
410 unit of β-galactopyranose, a unit of α-glucopyranose, and four units of β-fructofuranose.
411 Following the same procedure, as for compound **B**, the position of glycosidic linkages
412 was analyzed from the gHMBC spectra. For **C** and **D** the same relevant correlations
413 than in compound **B** were observed. In addition, one and two additional correlations
414 between β-Fru-H1 methylene protons and the anomeric carbon of the following β-Fru
415 unit were observed for **C** and **D** respectively. So the second most abundant LFOS (peak

11, **Fig. 2**) was identified as the pentasaccharide β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside (**Fig. 5C**). Finally, the structure of peak 13 (**Fig. 2**) was unequivocally elucidated as β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside (**Fig. 5D**). To the best of our knowledge, these data provide the first evidence of ^{1}H and ^{13}C NMR full assignments for this hexasaccharide.

Taking into account the enzyme´s mechanism of action described for the synthesis of compounds **B**, **C**, and **D**, these results led us to tentatively determine that peaks 15 and 17 (**Fig. 2**) correspond with structures **E** and **F** (**Fig. 5**), established as β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside and β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside, respectively.

4. Conclusions

This work describes a novel bi-enzymatic system catalyzed by the levansucrase from *Bacillus subtilis* CECT 39 (LS) along with the inulosucrase enzyme from *Lactobacillus gasseri* DSM 20604 (IS) for the efficient synthesis of lactosyl-oligofructosides (LFOS) using the disaccharides sucrose and lactose as substrates. LFOS consist of a lactosucrose moiety that is elongated by a linear chain of fructosyl residues bound by β-(2→1) linkages. The main formed LFOS were those of DP 4 to 6 (mono-, di-, and trifructosyl-lactosucrose) although other LFOS with higher DP (up to DP 8) were also synthesized. To the best of our knowledge, LFOS of DP 6-8 are

441 described for the first time in this work. The synthesis of LFOS can also be
442 straightforwardly addressed from sucrose:lactosucrose mixtures catalyzed by IS
443 reaching yields of up to 81% (in weight, respect to the initial amount of lactosucrose).
444
445 ***Acknowledgments***
446 This work has been financed by project AGL2011-27884 from Ministerio de
447 Ciencia e Innovación. M. Díez-Municio is supported by CSIC through JAE-Pre
448 Programme co-funded by ESF. M. Herrero thanks MICINN for his "Ramón y Cajal"
449 contract. The authors gratefully acknowledge Dr. A. Gonçalves for the cloning of IS.
450

451 ***References***

452 - Anwar, M. A., Kralj, S., Piqué, A. V., Leemhuis, H., van der Maarel, M. J. E. C., &
453 Dijkhuizen, L. (2010). Inulin and levan synthesis by probiotic *Lactobacillus gasseri*
454 strains: characterization of three novel fructansucrase enzymes and their fructan
455 products. *Microbiology, 156*, 1264-1274.

456 - Canedo, M., Jimenez-Estrada, M., Cassani, J., López-Munguia, A. (1999). Production
457 of maltosylfructose (erlose) with levansucrase from *Bacillus subtilis*. *Biocatalysis and*
458 *Biotransformation*, *16*, 475-485.

459 - Côté, G. L. (2007). Flavorings and other value-added products from sucrose. In R.A.
460 Rastall, (Ed.), *Novel enzyme technology for food applications,* (pp. 243-269). Boca
461 Ratón, FL: CRC Press.

462 - Curiel, J. A., de las Rivas, B., Mancheño, J. M., & Muñoz, R. (2011). The pURI
463 family of expression vectors: a versatile set of ligation independent cloning plasmids for
464 producing recombinant His-fusion proteins. *Protein Expression and Purification, 76*,
465 44-53.

466 - Davies, G. J., Wilson, K. S., & Henrissat, B. (1997). Nomenclature for sugar-binding
467 subsites in glycosyl hydrolases. *Biochemical Journal, 321*, 557-559.

468 - Díez-Municio, M., de las Rivas, B., Jimeno, M. L., Muñoz, R., Moreno, F. J., &
469 Herrero, M. (2013). Enzymatic synthesis and characterization of fructooligosaccharides
470 and novel maltosylfructosides by inulosucrase from *Lactobacillus gasseri* DSM 20604.
471 *Applied and Environmental Microbiology, 79*, 4129-4140.

472 - Díez-Municio, M., Herrero, M., Olano, A., & Moreno, F. J. (2014). Synthesis of novel
473 bioactive lactose-derived oligosaccharides by microbial glycoside hydrolases. *Microbial*
474 *Biotechnology, 7*, 315-331.

- Fujita, K., Hara, K., Hashimoto, H., & Kitahata, S. (1990). Transfructosylation catalyzed by β-fructofuranosidase I from *Arthrobacter sp.* K-1. *Agricultural and Biological Chemistry, 54*, 2655-2661.

- Fujita, K., Hara, K., Hashimoto, H., & Kitahata, S. (1992). Method for the preparation of fructose-containing oligosaccharide. US Patent 5,089,401.

- Fujita, K., Kishino, E., Fukuhara, I., Takehara, I., Ikeda, H., & Ito, T. (2006). Effects of single and two-week repeated ingestion of granules containing lactosucrose on calcium excretion in healthy males. *Journal of Japanese Council for Advanced Food Ingredients Research, 9*, 46-56.

- Fujita, K., Ito, T., & Kishino, E. (2009). Characteristics and applications of lactosucrose. *Proceedings Research Society of Japan Sugar Refineries Technologists*, 657, 13-21.

- Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews, 17*, 259-275.

- Han, W. -C., Byun, S. -H., Kim, M.-H., Sohn, E. H., Lim, J. D., Um, B. H., Kim, C. H., Kang, S. A., & Jang, K. -H. (2009). Production of lactosucrose from sucrose and lactose by a levansucrase from *Zymomonas mobilis*. *Journal of Microbiology and Biotechnology, 19*, 1153-1160.

- Harmsen, H. J. M., Raangs, G. C., Franks, A. H., Wildeboer-Veloo, A. C. M., & Welling, G. W. (2002). The effect of the prebiotic inulin and the probiotic *Bifidobacterium longum* on the fecal microflora of healthy volunteers measured by FISH and DGGE. *Microbial Ecology in Health and Disease*, 14, 211-219.

- Li, W., Xiang, X. L., Tang, S. F., Hu, B., Tian, L., Sun, Y., Ye, H., & Zeng, X. (2009). Effective enzymatic synthesis of lactosucrose and its analogues by β-D-galactosidase from *Bacillus circulans*. *Journal of Agricultural and Food Chemistry, 57*, 3927-3933.

- Moreno, F. J., Díez-Municio, M., Herrero, M., Corzo, N., Muñoz, R., de las Rivas, B., & Jimeno, M. L. (2014). Procedimiento bi-enzimático de síntesis eficiente de oligosacáridos fructosilados derivados de lactosacarosa, productos obtenidos y su uso en la mejora de la salud gastrointestinal. *ES Pat.*, P201430784.

- Mu, W., Chen, Q., Wang, X., Zhang, T., & Jiang, B. (2013). Current studies on physiological functions and biological production of lactosucrose. *Applied Microbiology and Biotechnology, 97*, 7073-7080.

- Ohkusa, T., Ozaki, Y., Sato, C., Mikuni, K., & Ikeda, H. (1995). Long-term ingestion of lactosucrose increases *Bifidobacterium* sp. in human fecal flora. *Digestion, 56*, 415-420.

- Ozimek, L. K., Kralj, S., van der Maarel, M. J., & Dijkhuizen, L. (2006). The levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121 catalyse processive and non-processive transglycosylation reactions. *Microbiology, 152*, 1187-1196.

- Park, N. H., Choi, H. J., & Oh, D. K. (2005). Lactosucrose production by various microorganisms harboring levansucrase activity. *Biotechnology Letters, 27*, 495-497.

- Perrin, S., Fougnies, C., Grill, J. P., Jacobs, H., & Schneider, F. (2002). Fermentation of chicory fructo-oligosaccharides in mixtures of different degrees of polymerization by three strains of bifidobacteria. *Canadian Journal of Microbiology, 48,* 759-763.

- Pijning, T., Vujičić-Žagar, A., Kralj, S., Dijkhuizen, L., & Dijkstra, B. W. (2012). Structure of the α-1, 6/α-1, 4-specific glucansucrase GTFA from *Lactobacillus reuteri* 121. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, *68*, 1448-1454.

- Pilgrim, A., Kawase, M., Ohashi, M., Fujita, K., Murakami, K., & Hashimoto, K. (2001). Reaction kinetics and modelling of the enzyme-catalyzed production of lactosucrose using β-fructofuranosidase from *Arthrobacter sp.* K-1. *Bioscience, Biotechnology and Biochemistry, 65*, 758-765.

- Seibel, J., Moraru, R., Götze, S., Buchholz, K., Na'amnieh, S., Pawlowski, A., & Hecht, H. J. (2006). Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase). *Carbohydrate Research, 341*, 2335-2349.

- Teramoto, F., Rokutan, K., Sugano, Y., Oku, K., Kishino, E., Fujita, K., Hara, K., Kishi, K., Fukunaga, M., & Morita, T. (2006). Long-term administration of 4G-β-D-galactosylsucrose (lactosucrose) enhances intestinal calcium absorption in young women: a randomized, placebocontrolled 96-wk study. *Journal of Nutritional Science and Vitaminology (Tokyo), 52*, 337-346.

- Tuohy, K. M., Finlay, R. K., Wynne, A. G., & Gibson, G. R. (2001). A human volunteer study on the prebiotic effects of HP-inulin-faecal bacteria enumerated using fluorescent in situ hybridisation (FISH). *Anaerobe, 7*, 113-118.

- Vandamme, E. J. (2009). Agro-industrial residue utilization for industrial biotechnology products. In P. S. nne' Nigam & A. Pandey (Eds.), *Biotechnology for Agro-Industrial Residues Utilisation. Utilisation of Agro-Residues,* (pp. 3-11). Germany: Springer.

- Yamamori, A., Onodera, S., Kikuchi, M., & Shiomi, N. (2002). Two novel oligosaccharides formed by 1^F-fructosyltransferase purified from roots of asparagus (*Asparagus officinalis* L.). *Bioscience, Biotechnology, and Biochemistry, 66*, 1419-1422.

548 ***Figure captions***

549

550 **Figure 1.** Concentrations of sucrose, lactose, total fructo-oligosaccharides (FOS)
551 lactosucrose and other fructosylated oligosaccharides derived from lactose (LFOS),
552 upon transfructosylation reaction catalyzed by inulosucrase from *Lactobacillus gasseri*
553 DSM 20604 (IS) (1.6 U mL^{-1}) in the presence of sucrose:lactose (A) or
554 sucrose:lactosucrose (B) mixtures (250 g L^{-1} each subtrate) in 25 mM sodium acetate
555 buffer with 1 mM $CaCl_2$ (pH 5.2) at a temperature of 55 ºC. Vertical bars represent
556 standard deviations (n = 3).

557

558 **Figure 2**. LC-RID profile after 24 h of transfructosylation reaction based on
559 sucrose:lactosucrose (250 g L^{-1} each) catalyzed by inulosucrase from *Lactobacillus*
560 *gasseri* DSM 20604 (IS) (1.6 U mL^{-1}) at 55 °C, in 25 mM sodium acetate buffer
561 supplemented with 1 mM $CaCl_2$ (pH 5.2), where lactosyl-oligofructosides (LFOS)
562 synthesis is observed in high yield and percent purity. Peak identification: 1, fructose; 2,
563 glucose; 3, sucrose; 4, inulobiose; 5, lactose; 6, 1-kestose; 7, lactosucrose; 8, nystose
564 (GF3); 9, 11, 13, 15, 17, LFOS DP 4 - 8; 10, 12, 14, 16, fructo-oligosaccharides DP 5 -
565 8 (GF4-7).

566

567 **Figure 3.** Effect of enzyme charge, pH and temperature on the concentration of
568 lactosucrose formed by transfructosylation reaction catalyzed by levansucrase from
569 *Bacillus subtilis* CECT 39 in 50 mM potassium phosphate buffer. A) Effect of enzyme
570 charge (0.25 and 0.5 U mL^{-1}) at 37ºC and pH 6. B) Effect of pH (5.0, 6.0 and 7.0) at
571 37ºC using 0.5 U mL^{-1} of enzyme. C) Effect of temperature (30, 37, 45 and 55°C) at pH
572 6 using 0.5 U mL^{-1} of enzyme. Vertical bars represent standard deviations (n = 3).

573

574 **Figure 4.** Process scheme for the synthesis of lactosyl-oligofructosides (LFOS) by
575 enzymatic transfructosylation of lactosucrose catalyzed by an inulosucrase from
576 *Lactobacillus gasseri* DSM 20604 using the disaccharides sucrose and lactose as
577 substrates previous synthesis of lactosucrose with a levansucrase from *Bacillus subtilis*
578 CECT 39 through a bi-enzymatic system (A) or by using sucrose and lactosucrose as
579 starting materials (B).

580

581 **Figure 5.** Structure of lactosucrose (A) and the synthesized lactosyl-oligofructosides
582 (LFOS) products (B - F) based on sucrose:lactose catalyzed by inulosucrase from
583 *Lactobacillus gasseri* DSM 20604 (IS) upon transfructosylation reaction.
584 A) Lactosucrose: β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-Fru; B) LFOS DP4: β-D-Gal-
585 (1→4)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru; C) LFOS DP5: β-D-Gal-(1→4)-α-D-
586 Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru; D) LFOS DP6: β-D-Gal-(1→4)-
587 α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru; E) LFOS
588 DP7: β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-
589 (1→2)-β-D-Fru-(1→2)-β-D-Fru; F) LFOS DP8: β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-
590 Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru-(1→2)-β-D-Fru.

Table 1. Concentration and relative percentages of the most-abundant synthesized lactosyl-oligofructosides (LFOS) from DP4 to DP7 and fructo-oligosaccharides (FOS) from DP3 to DP6 upon transfructosylation reaction in the presence of sucrose:lactosucrose or sucrose:lactose mixtures (250 g L^{-1} each substrate) under the optimum reaction conditions. Data shown as mean ± sd (n = 3).

	Sucrose:Lactosucrose[a]		Sucrose:Lactose[b]	
Synthesized compounds	**Concentration (mg mL^{-1})**	**Relative Perc.[c] (%)**	**Concentration (mg mL^{-1})**	**Relative Perc.[c] (%)**
LFOS	202.7	80.5	54.2	83.0
DP4 (Peak 9, Fig. 2)	97.6 ±4.64	38.8	37.9 ±0.97	58.0
DP5 (Peak 11, Fig. 2)	66.0 ±3.44	26.2	12.4 ±1.12	19.0
DP6 (Peak 13, Fig. 2)	30.8 ±1.76	12.2	3.9 ±0.05	6.0
DP7 (Peak 15, Fig. 2)	8.3 ±0.49	3.3		
FOS	49.1	19.5	11.1	17.0
DP3 (Peak 8, Fig. 2)	18.7 ±0.86	7.4	8.9 ±0.22	13.6
DP4 (Peak 10, Fig. 2)	17.0 ±1.06	6.8	2.2 ±0.14	3.4
DP5 (Peak 12, Fig. 2)	8.3 ±0.43	3.3		
DP6 (Peak 14, Fig. 2)	5.1 ±0.11	2.0		
TOTAL	251.8		65.3	

[a] Reaction catalyzed by inulosucrase from *Lactobacillus gasseri* DSM 20604 (IS) under optimized conditions described in section 2.4.

[b] Applying a new bi-enzymatic system based on *Bacillus subtilis* CECT 39 levansucrase (LS) -*Lactobacillus gasseri* DSM 20604 inulosucrase (IS) . Optimized reaction conditions are described in section 3.2.

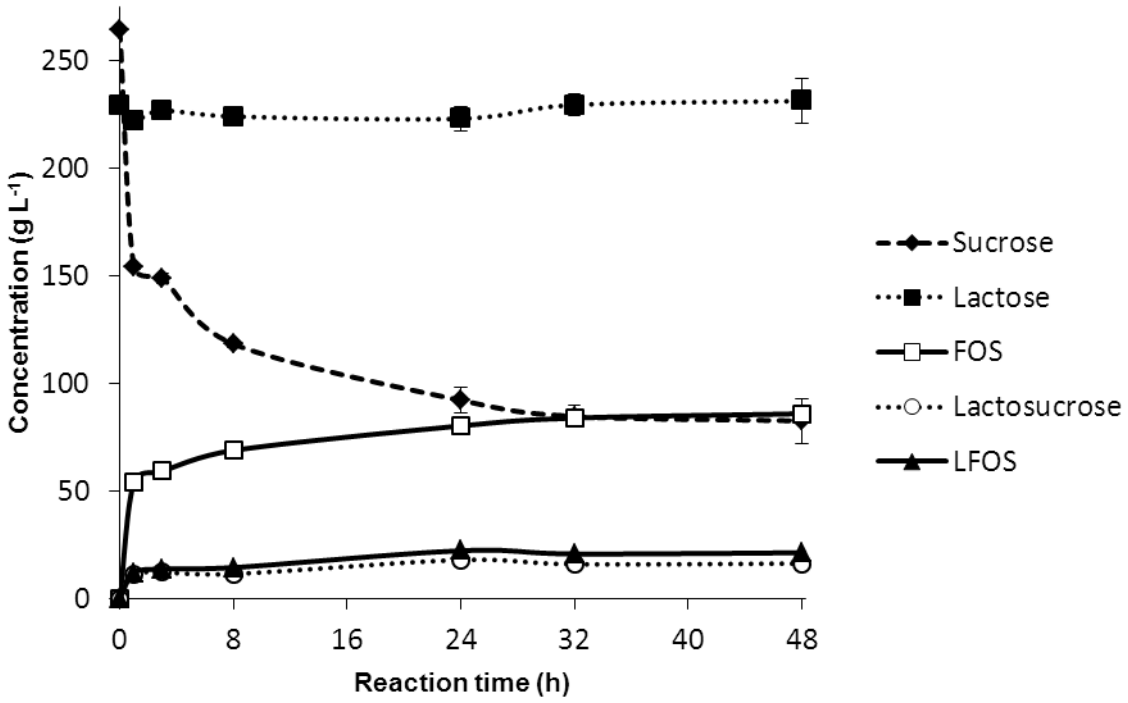
[c] Relative percentages (%) data were calculated with respect to the total synthesized compounds values shown in the entries in the bottom row of the table.

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for oligosaccharides **B-D**. Chemical shift (δ, ppm) and coupling constants (J in Hz, in parentheses).

Structure	Position	Gal		Glc		Fru 1		Fru 2		Fru 3		Fru 4	
		δ_H	δ_C	δ_H	δ_H	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
B β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.33 (7.9)	105.67	5.28 (3.9)	94.99	3.69 3.60	63.58	3.59 3.53	63.19				
	2	3.41	73.75	3.45	73.57	--	106.04	--	106.49				
	3	3.53	75.30	3.74	73.98	4.13	79.33	4.04	79.38				
	4	3.78	71.33	3.56	80.81	3.89	76.59	3.94	77.22				
	5	3.59	78.13	3.84	73.84	3.73	84.00	3.72	83.88				
	6	3.67 3.62	63.80	3.76 3.70	62.26	3.66 3.62	64.91	3.69 3.63	65.07				
C β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.33 (7.8)	105.67	5.28 (3.9)	95.00	3.70 3.60	63.75	3.72 3.59	63.64	3.61 3.54	63.12		
	2	3.41	73.75	3.45	73.60	--	106.00	--	105.80	--	106.42		
	3	3.53	75.30	3.74	73.99	4.13	79.50	4.08	80.24	4.04	79.47		
	4	3.78	71.33	3.56	80.81	3.89	76.60	3.93	77.19	3.96	77.07		
	5	3.59	78.13	3.84	73.85	3.73	84.00	3.71	83.82	3.72	83.82		
	6	3.67 3.62	63.80	3.75 3.72	62.27	3.68 3.65	64.92	3.70 3.67	64.99	3.71 3.69	64.99		
D β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	1	4.33 (7.8)	105.67	5.29 (3.9)	95.00	3.70 3.59	63.71	3.72 3.59	63.40	3.72 3.59	63.68	3.61 3.54	63.20
	2	3.41	73.75	3.45	73.62	--	106.00	--	105.81	--	105.79	--	106.45
	3	3.53	75.30	3.74	73.99	4.13	79.48	4.09	80.08	4.07	80.28	4.04	79.48
	4	3.78	71.33	3.57	80.81	3.89	76.60	3.95	77.03	3.93	77.07	3.96	77.20
	5	3.59	78.13	3.84	73.86	3.73	84.02	3.72	83.83	3.71	83.83	3.71	83.83
	6	3.67 3.62	63.80	3.75 3.72	62.28	3.67 3.64	64.94	3.67 3.60	64.94	3.67 3.62	64.94	3.69 3.62	65.02

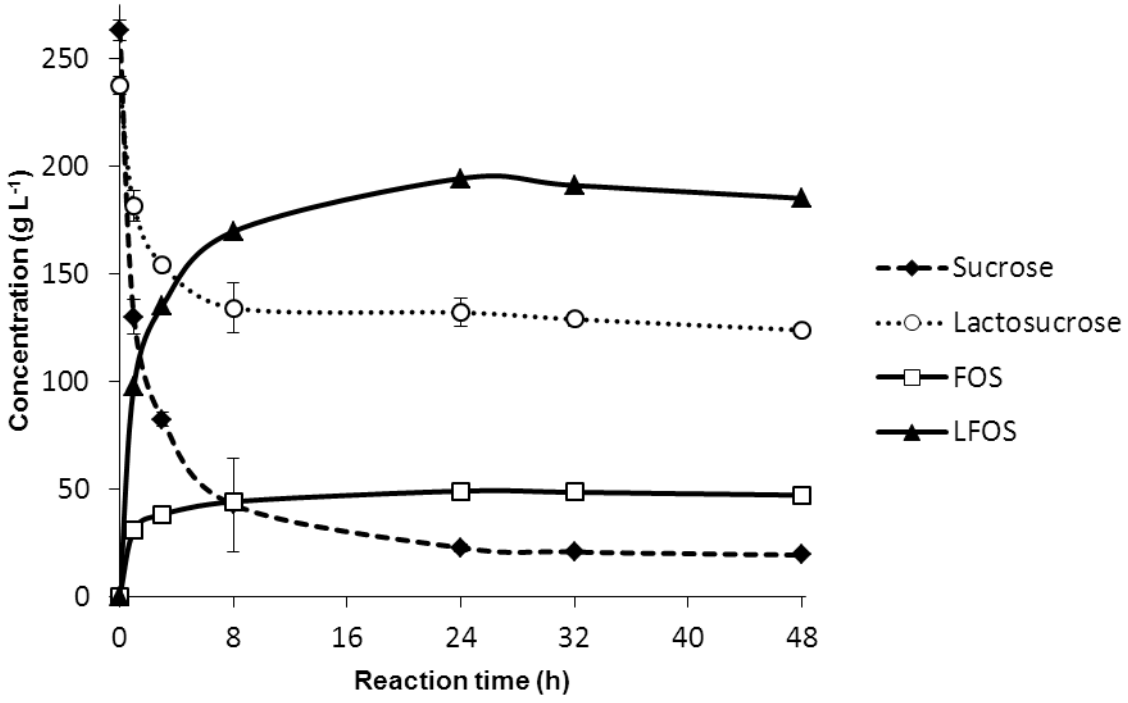
606 **Figure 1.** *Díez-Municio et al.*

607 **A)**



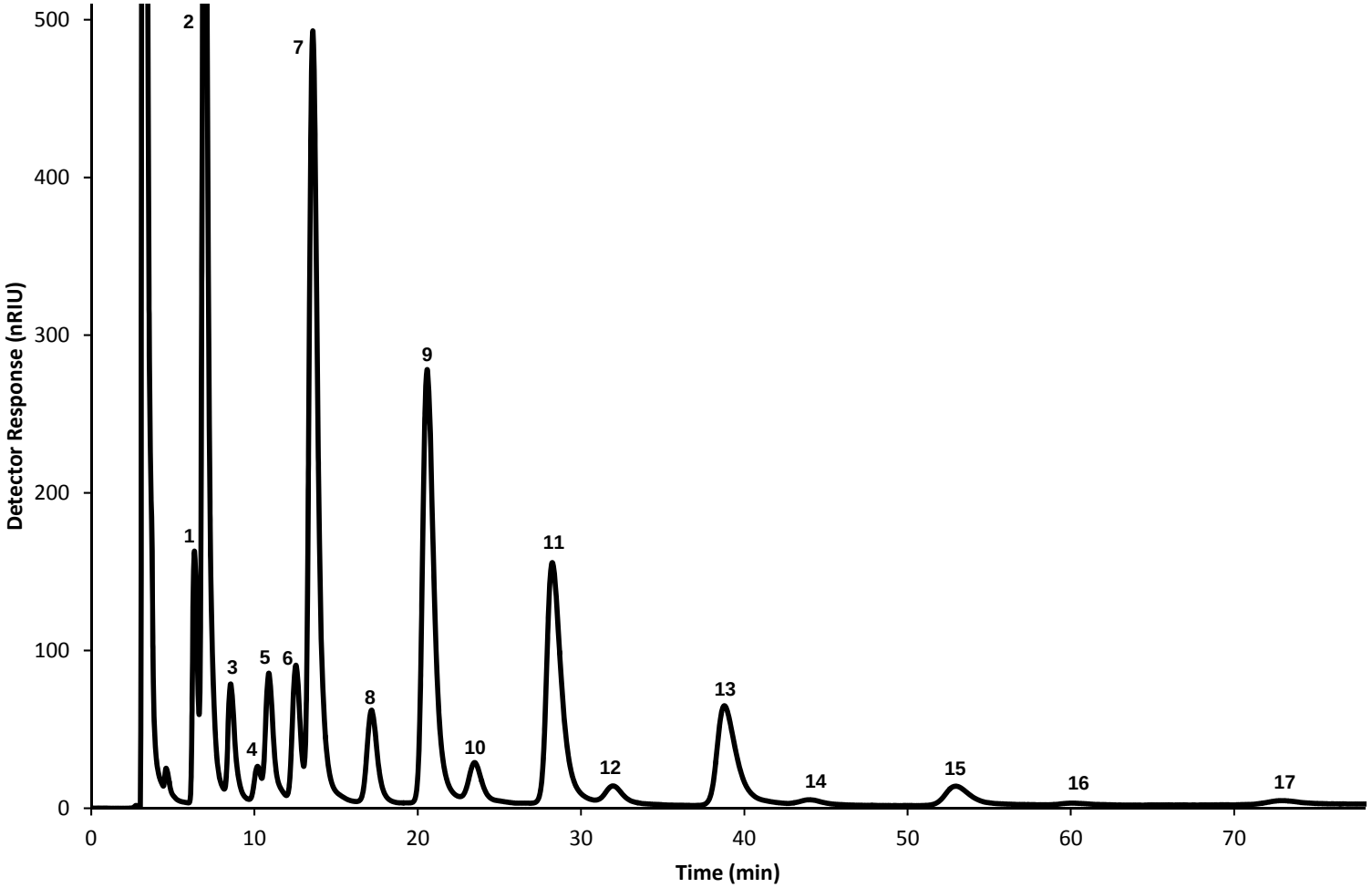
608

609 **B)**



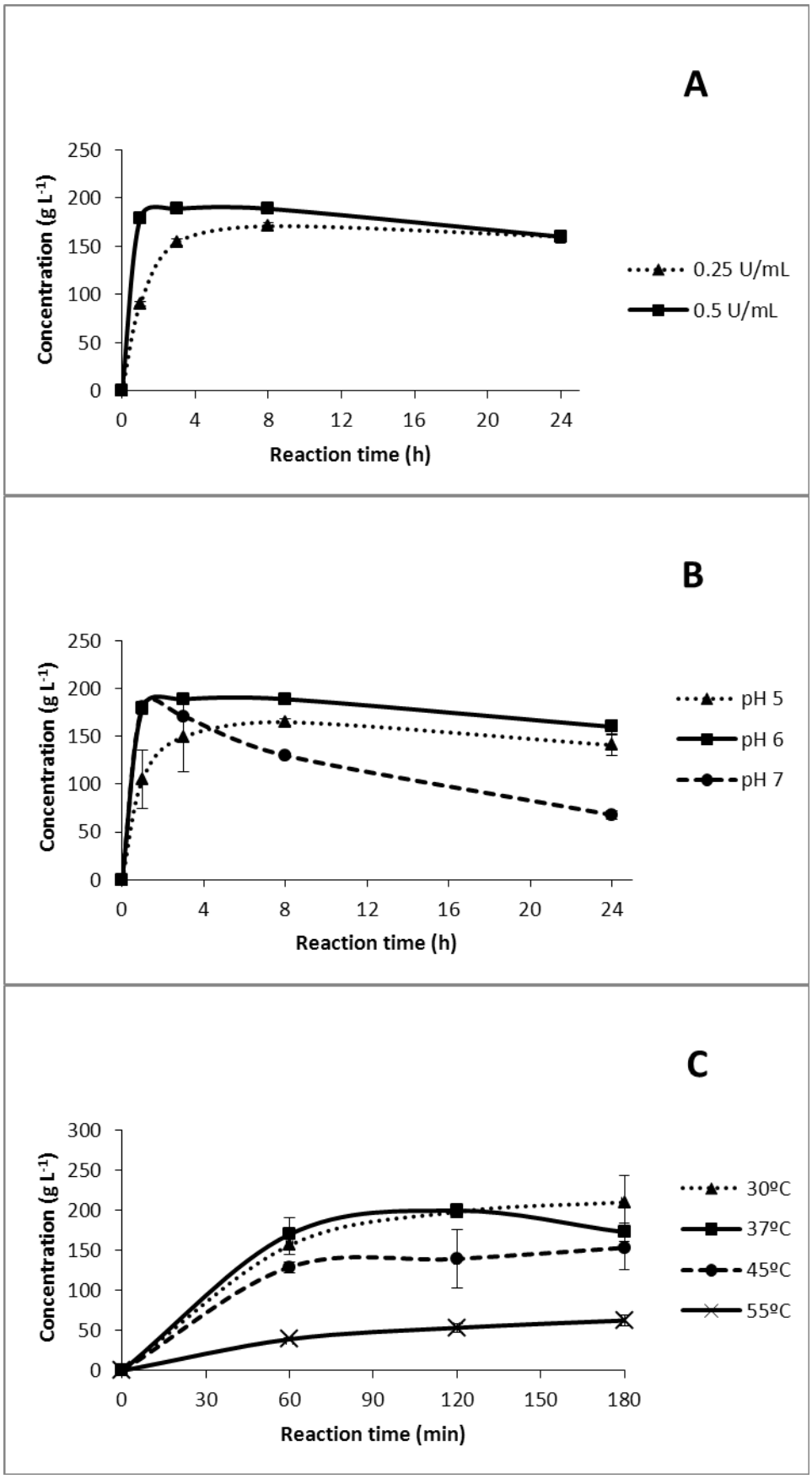
610

611 **Figure 2.** *Díez-Municio et al.*



612

613 **Figure 3.** *Díez-Municio et al.*

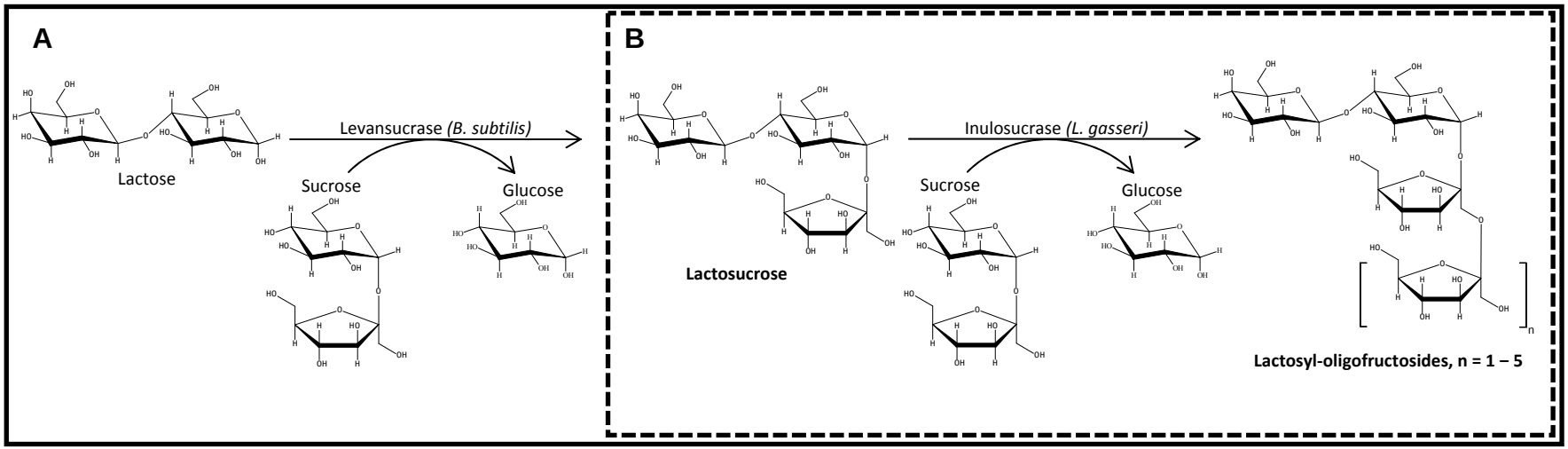


614

615

616

Figure 4. *Díez-Municio et al.*



626 **Figure 5.** *Díez-Municio et al.*

627

628